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Pollen Embryogenesis to Induce, Detect, and Analyze Mutants

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The development of fully differentiated plants from individual pollen grains through a series **of developmental phases that resemble embryogenesis beginning with the zygote was demonstrated during the mid-1960's. This technology opened the door to the use of haploid plants (sporophytes with the gametic number of chromosomes) for plant breeding and genetic studies, biochemical and metabolic studies, and the selection of mutations. Although pollen embryogenesis has been demonstrated successfully in numerous plant genera, the procedure cannot as yet be use routinely to generate large populations of plants for experiments. Practical results from use of the technology in genetic toxicology research to detect mutations have failed to fully realize the theoretical potential; further developments of the technology could overcome the limitations. Pollen embryogenesis could be used to develop plants from mutant pollen grains to verify that genetic changes are involved. Through either spontaneous or induced chromosome doubling,these plants can be made homozygous and used to analyze genetically the mutants involved. The success of this approach will depend on the mutant frequency relative to the fraction of pollen grains that undergo embryogenesis; these two factors will dictate population size needed for success. Research effort is needed to further develop pollen embryogenesis for use in the detection of genotoxins under both laboratory and *in situ* conditions.**

Introduction

Plant cells, except for those few kinds that have undergone irreversible differentiation, are totipotent; i.e., a single cell can express its full genetic potential by differentiating and developing into a complete plant through embryo-like stages. For this to occur *in vitro*, certain requirements must be met for a source of energy, essential mineral elements, vitamins, hormones, light quality, light intensity, photoperiod, and temperature in an aseptic environment of the proper atmosphere. The specific requirements vary among species. Somatic (body) cells are capable of expressing the genotype of the individual from which they were derived in a similar manner and to the same extent through embryogenesis *in vitro* as zygotes through embryogenesis *in vivo*. In gametophytic (reproductive) cells that possess only the haploid number of chromosomes, totipotency *in vitro* leads to the development of plants with, at least in theory, only half of the somatic number of chromosomes in their cells. Refer to Raghavan (*1*) for a discussion of the concept of totipotency in general and of totipotency in somatic and reproductive cells.

In this report, pollen embryogenesis will be defined; procedures for obtaining pollen embryogenesis that are commonly used will be described; developmental pathways involved in pollen embryogenesis will be discussed; and the potential use of pollen embryogenesis to induce, detect, and/or to genetically characterize mutants will be discussed. The primary emphasis will be placed on the potential role of this technology relative to the use of plants to determine the mutagenicity of chemical substances in our environment. Primarily, information regarding angiospermous plants will be presented.

Pollen Embryogenesis

The term pollen embryogenesis will be used in the broad sense to include the development of plants with the gametic number of chromosomes from haploid cells of the microgametophyte (pollen grain). In this context,the term haploid denotes the

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reduced number of chromosomes that results from meiosis. Implicit in this terminology is that plant development from pollen grains progresses through a series of stages that are similar to those observed in embryogenesis *in vivo* beginning from the zygote (the fertilized egg cell). I am including, however, the development of plants through organogenesis from callus derived from *in vitro* cultured-pollen grains. Both routes to plant development are potentially valuable.

Microsporogenesis and Microgametogenesis

The details concerning microsporogenesis and microgametogenesis vary considerably among genera. In general, the processes involve the development of four microspores, each having half (N) the somatic number of chromosomes from one microsporocyte (pollen mother cell). Each microsporocyte has $2N$ number of chromosomes. The microspores develop into the mature pollen grains that produce the sperm cells (nuclei).

In the angiospermous plants, the pollen mother cells develop in the microsporangia (pollen sac) of the anther. The pollen mother cells have the $2N$ number of chromosomes and the corresponding $2C$ amount of DNA that doubles to the $4C$ amount prior to meiotic prophase. The pollen mother cells then undergo the various stages (i.e., leptonema, zygonema, pachynema, diplonema and diakinesis) of the prolonged meiotic prophase I followed by metaphase I, anaphase I, and telophase I. At that point a binucleate cell is formed whose individual nuclei have the N number of chromosomes occurring as bivalents (each chromosome has two chromatids) with the corresponding $2C$ amount of DNA. A short interphase follows without DNA synthesis. The second meiotic division proceeds much as in mitosis except that each nucleus has only half as many chromosomes involved as are in somatic cells. This division technically separates the chromatids and yields four microspores arranged in a tetrad; each microspore has the N number of chromosomes and the $1C$ amount of DNA.

Generally, the microspore is involved in two mitotic divisions that form the multinucleate microgametophyte (pollen grain). In the course of development, the pollen grains dissociate themselves from the callose matrix of the tetrad and become thin-walled and spherical in shape. The tough, outer wall (exine) and the inner, cellulosic wall (intine) are formed. A large vacuole forms that generally pushes the nucleus next to the intine at one end of the pollen grain. DNA synthesis occurs in the nucleus in preparation for the first pollen mitosis, which forms the generative and vegetative cells (commonly referred to as nuclei).

The vegetative (tube) nucleus is the larger of the two nuclei formed by the first pollen mitosis. It stains lightly with nucleic acid stains, is highly metabolically active, encloses various organelles and vacuoles, and does not normally undergo another mitosis. In contrast, the generative nucleus is the smaller one, is generally observed close to the intine at one end of the pollen grain, stains darkly with nucleic acid stains, encloses few organelles in a small volume of cytoplasm, and will divide once more forming the two sperm nuclei. Generally the generative nucleus arrests in prophase of the second pollen grain mitosis at which stage it can remain for an extended period of time depending on the species.

In those species with trinucleate pollen grains, DNA synthesis occurs in the generative nucleus followed by the second pollen mitosis before anthesis. In those species with binucleate pollen grains, it appears that DNA synthesis occurs in the generative nucleus prior to anthesis, while the second pollen mitosis occurs after pollination during tube growth. Brewbaker and Emery (*2*) Sunderland (*3, 4*), Reinert and Bajaj (*5*), Cass (*6*), Mascarenhas (*7*), and Cass and Karas (*8*) have given greater details relating to microsporogenesis and microgametogenesis.

Historical Perspectives of Pollen Embryogenesis

Callus was successfully cultured *in vitro* from pollen of *Ginkgo biloba* during the early 1950's (*9*); however, plant development from the haploid callus was not achieved. Tulecke (*10*) was able to isolate spontaneous arginine-requiring "mutant" callus by culturing pollen of Ginkgo on medium enriched with L-arginine, thereby indicating the potential of pollen embryogenesis in mutagenesis research. The first successful embryo development from pollen grains was achieved in *Datura innoxia* in 1964 by Guha and Maheshwari (*11*). Three years later, they reported the development of haploid plants from pollen grains of Datura (*12*). Bourgin and Nitsch (*13*) in 1967 also described the development of haploid plants from pollen grains of Nicotiana. Reports of successes in other genera followed quickly thereafter; however, not all genera have been successfully cultured from pollen grains to plants. Even in those in which culture has been achieved, the procedure is neither uniformly nor routinely

successful in producing large numbers of plants from each anther.

Pathways in Pollen Embryogenesis

This section will present a brief and general discussion of pathways involved in pollen embryogenesis based primarily on studies involving Nicotiana and Datura. Please refer to Sunderland (*3, 4*), Sunderland and Dunwell (*14*), Reinert and Bajaj (*5*), Nitsch (*15, 16*) Clapham (*17*), Sink and Padmanabhan (*18*), and Collins (*19*) for more details.

The two major pathways from pollen grains to plants are direct and indirect androgenesis (*5*). The direct pathway involves microspore differentiation through a series of stages that simulate embryogenesis *in vivo* from the zygote. Globular stage embryos generally are released from the pollen grain wall, they develop through the heart and torpedo stages, and finally the cotyledons unfold and the plant emerges from inside the anther wall. The process requires from 4 to 8 weeks. The indirect pathway involves the formation of a callus from the microspore, which bursts through the anther wall, and then differentiates to form either embryos or roots and shoots (organogenesis). Plant development via this pathway is somewhat slower and the range of ploidy of the plants recovered is greater than via the direct pathway. Thus, the direct pathway is generally more desirable than the indirect one. This is especially true for any application of the technology in the context of genetic toxicology.

The induction principle that initiates microspores to alter their normal developmental path to one involving continued cell division and differentiation to form plants, is not fully understood. Numerous factors are known to affect both induction and continuation of embryogenesis, and they frequently do so through interactions. Among the factors are: physiological status of the mother plant, developmental stage of the pollen, shock treatment of the pollen, environmental conditions during culture, and media composition (e.g., amino acids, growth regulators, sucrose concentration, form and amount of nitrogen, and activated charcoal). Plant species vary considerably in their response to these various factors. For example, Datura and Nicotiana require only a sucrose solution as a medium. They do respond positively, however, to a number of factors by producing a greater percentage of anthers with plants and a greater number of plants per productive anther. Embryogenesis has been induced by the culture of pollen grains beginning as early as the tetrad stage. The uninucleate pollen grain, however, is usually the preferred stage, and embryogenetic potential virtually disappears after starch accumulation occurs.

Three developmental patterns involving the generative and vegetative nuclei in cultured pollen grains are recognized to be significant to pollen embryogenesis (*3, 4, 14*). Type A pattern involves repeated divisions of the vegetative nucleus until the exine ruptures permitting the embryoid to continue its development inside the microsporangium. If the generative cell divides, it does so for a limited number of times and the cells formed do not contribute to the embryoid. Type B pattern involves a symmetrical division of the microspore into two equally diffuse nuclei, each resembling a vegetative nucleus. One or both of these nuclei can undergo further division and differentiation to form an embryoid. It is suspected that in some cases the two nuclei fuse to form a diploid embryo rather than the expected haploid condition. Type C pattern involves a normal asymmetric division of the microspore into a generative and vegetative nucleus. This pattern differs from Type A, in that the generative nucleus contributes to the formation of the embryoid. Although this pattern has been observed only in *Datura innoxia*, it is suspected to be operative in other species in which nonhaploid embryoids are produced.

Procedures Generally Used in Pollen Embryogenetic Studies

Since numerous factors contribute towards successful embryogenesis from pollen grains and plant species differ in response to these factors, procedures for successful embryogenesis will vary. General procedures to be used for either anther or pollen grain culture using either solid or liquid media will be described. Obviously, several modifications of these procedures might have to be tried to optimize embryogenetic conditions for each species.

1. The mother plants should be grown under optimum environmental conditions and should be free of insect pests and diseases. Certain genetic strains and varieties respond more favorably than others. Light quality, intensity, and duration are important factors in the culture of mother plants.

2. Flower buds should be collected when pollen grains are uninucleate, unless the species involved is known to respond better at another pollen developmental stage. Pollen developmental stage must be determined, preferably by establishing a correlation between visual characteristics of the bud and the various developmental stages of the pollen

grains. For example, when the corolla extends slightly beyond the tip of the calyx in *Nicotiana tabacum*, the pollen grains are generally uninucleated — the desired stage for induction of embryogenesis. Not all species are as easy as *Nicotiana tabacum* to determine pollen grain developmental stages.

3. A shock such as chilling for several days or removal of tillers from the mother plant to water culture will frequently improve the embryogenetic response observed when the anthers or the pollen grains are placed in medium under proper environmental conditions.

4. The flower buds are generally surface-sterilized with sodium hypochlorite or another suitable sterilizing solution. From then on, all operations should be done under sterile conditions.

5. The flower buds are then opened and the anthers are removed. (It is usually advisable to handle the anthers by means of the filaments to prevent mechanical damage.)

6. Anthers are then placed either on the surface of solid medium or floated on the surface of a shallow layer of liquid medium. (Care must be taken to avoid severe agitation that would cause the anthers to sink.)

7. Several media are known to be suitable for pollen embryogenesis, and the investigator should either try various modifications of these or select one based on information in the literature. [Refer to Reinert and Bajaj (*5*), for example, for a discussion of media formulations.]

8. Anthers are generally cultured in the dark at 22-27°C for several days during which time embryogenesis is initiated. Anthers should be examined periodically for evidence of induction of embryogenesis by a staining reaction for nucleic acids.

9. At the completion of the induction period, the anthers must be cultured in the light until plants develop sufficiently to be transplanted. An alternate procedure is to mechanically rupture the anthers at that time, isolate the pollen grains from the debris by filtration and centrifugation, and culture the free pollen grains in liquid medium. One should inoculate 2.5 ml of liquid medium in a small petri plate with 2.5×10^4 pollen grains.

10. After a period of 4-8 weeks in culture, green plants should be available for transplanting to a growth medium *in vitro* for further growth and development and later transplanted to a potting mix under mist. All plants should be analyzed cytologically to verify their chromosome number.

11. In those species where embryogenesis occurs via callus derived from pollen grains, the procedure described above must be modified somewhat. For example, after the callus bursts the anther wall, the callus must be transferred to a different medium that will induce and support either embryogenesis or organogenesis. Plants are then removed from the callus and handled in a manner similarly to that described in step 10 above. In some cases, rooting might have to be induced on a separate medium. More details regarding procedures have been published (*3-5, 14-17, 19*).

Pollen Embryogenesis to Induce and Detect Mutations

Pollen embryogenesis offers the potential to facilitate the induction and detection of mutations. Advantages include the following: large populations of individuals per anther; individuals exist as single cells; individuals have the gametic number of chromosomes; recessive genes as well as dominant ones may be expressed; doubling the chromosome number leads to homozygosity; and a high degree of developmental synchrony exists in certain species.

Most plans produce ample numbers of pollen grains per anther to fulfill the requirements of population size for experimental purposes. For example, a single *Nicotiana tabacum* anther has approximately 30,0000 pollen grains (*20*); *Zea mays* 3,100-3,700 (*21*), 6,000-7,500 (*22*); and *Nicotiana sylvestris*, 25,000 (Constantin, unpublished). Thus, a single plant can provide relatively large populations of individuals with which to work. From the tetrad stage of development onward, pollen grains exist as individuals. The reduced or gametic number of chromosomes exist from the microspore stage onward; however, DNA synthesis takes place in the microspore and in the generative and vegetative nuclei. In diploid species, recessive genes may be expressed in plants developed via pollen embryogenesis; however, in polyploid species and in those cases where multiple copies of a gene exist, the expression of traits can be complicated. The literature indicates that homozygous plants are obtained when chromosomes are doubled either through the use of colchicine or through the use of tissues whose cells undergo spontaneous endomitosis. This is important because it allows the recovery of a mutation in the homozygous state, which permits manipulations at the whole plant level.

Problems exist, however, including the following. Not all plant species respond positively to conditions generally used to induce and support continued embryogenesis from pollen grains. Even in the model species such as *Datura innoxia* and *Nicotiana tabacum*, only a relatively small fraction of the total population of pollen grains develop into plants. Although pollen grains exist singly, the

problem of chimerism is not completely circumvented because of DNA synthesis in the vegetative and generative nuclei. Procedures are available to assay for only a few traits expressed at the time of induction or even at the time the plants emerge from the anther wall. Thus, a considerable amount of time and space are required when the plants have to be grown to maturity. Lack of synchrony within the inflorescence and even within the anther leads to the treatment of a mixed population in terms of developmental stage. This can lead to complications in terms of interpretations including the determination of mutant frequency. Nonhaploid plants are frequently recovered, especially when the indirect route of androgenesis exists; i.e., via callus formation.

Little information is available in the literature on mutation induction and detection via the treatment of pollen followed by embryogenesis *in vitro*. Devreux and de Nettantcourt (*23*) have discussed the use of pollen embryogenesis in mutation induction. They described an experiment with *Nicotiana tabacum* in which mutants were recovered from irradiated microspores. Unfortunately, the selection procedure was not designed to select a specific mutant trait and the population size was inadequate to support strong conclusions. Sunderland (*3*) was unsuccessful in attempts to recover mutants of *Nicotiana tabacum* cv. White Burley after the treatment of excised anthers with *N*-methyl-*N*-nitrosoguanidine. Nitsch (*24*) added ethyl methylsulfonate to the medium on which *Nicotiana tabacum* cv. Wisconsin 38 anthers were cultured; one mutant plant was observed in a population of 137. He also reported that numerous mutants were observed from a population of plants derived from anthers exposed to 1500-3000 rads of γ-radiation. Although physical and chemical mutagens have been used successfully to induce mutations via pollen embryogenesis, the procedure has yet to be developed to the point of routine use as an assay for mutagenicity testing. For pollen embryogenesis to be effectively used in genetic toxicology, the following need to be achieved: develop the capability to routinely get large populations of plants from most, if not all, plated anthers of plant species with the requisite marker genes; develop biochemical screening procedures that can detect mutations affecting metabolism during early developmental phases of the plant; establish dose response curves for a series of chemicals with known mechanisms of mutagenesis administered at different stages of microsporogenesis, microgametogenesis, and pollen embryogenesis; and determine the role of DNA synthesis on the effects of various classes of chemicals expressed as mutants.

Pollen Embryogenesis to Verify and Analyze Mutants

Various plant genetic systems are being used to detect mutagens in the environment. In some cases, the exact genetics involved that lead to the observed phenotypic trait is not known. In other cases, the genetics of a particular trait is well understood; however, the mechanisms leading to an observed mutation involving that trait is not fully understood. Pollen embryogenesis could be used in those circumstances to improve our understanding of what is happening.

In the case of waxy locus in maize, the genetics involved is relatively well understood (*25-28*). However, more than one kind of genetic event can lead to the mutant expression of this trait. It would be helpful to know the distribution of mutants induced by different classes of agents according to the kinds of genetic events that were induced. To approach this problem via conventional hybridization techniques is a monumental task. Is the use of pollen embryogenesis within the realm of feasibility? Two important factors need to be considered. One is the mutant frequency observed following various mutagenic treatments, and the other is the fraction of pollen grains that undergo embryogenesis when cultured *in vitro*. In the maize inbred line, Early-Early Synthetic, the spontaneous mutant frequency is 10^{-5}. Plants treated repeatedly with ethyl methanesulfonate during their life cycles exhibit mutant frequencies that exceed 10^{-4} (*29*, *30*). These frequencies will vary depending on the genetic strains being used, and whether forward or reverse mutations are being analyzed.

Maize has been studied in terms of the development of plants through anther culture; however, pollen embryogenesis is far from being a routine procedure. For example, recent studies indicate that 1% or less of plated anthers respond positively and that positive-responding anthers yield only one embryoid each (*31*). One anther contains approximately 6000 pollen grains; therefore it is the rare individual grain that proceeds through embryogenesis *in vitro*. When one considers mutant frequency (10^{-5} to 10^{-4}) and positively responding anthers (10^{-2}) in maize, it is obvious that from 10^{6} to 10^{7} anthers would have to be cultured to recover a plant from a mutant pollen grain. It is evident that this approach is not a viable one.

It is logical then to ask the question: Is pollen embryogenesis a viable technique to be used with model plant species such as tobacco? Nitsch (*20*) has calculated that 7200 plants can be obtained per flower bud in *Nicotiana tabacum* with approximately

97% of these being haploid. Utilizing the same mutant frequency as in maize and assuming that 100% of the anthers would respond uniformly (in practice, a more realistic estimate would be from 10 to 33%), 15 flower buds would be required per mutant. Although it would not be an easy task to accumulate enough mutants to adequately represent all classes of genetic events, it is at least feasible. The primary limiting factor is that no genetic system involving tobacco pollen is available.

The status of the technology in terms of other species that have been and are being used to detect the mutagenicity of chemicals is somewhat between that in tobacco and that in maize. Barley, for example, has demonstrated only limited success (*32*), and I am unaware of any successful attempts at culturing pollen of Tradescantia.

In the case of mutations expressed in the M_2 generation from seeds, pollen embryogenesis might be useful in the analysis of genetic events. Pollen from plants of the treated population could be cultured to get haploid plants to be used for genetic analysis. The problems alluded to above would be encountered here also.

Conclusions and Recommendations

In theory, pollen embryogenesis offers great potential for use in the detection of genotoxins both in the laboratory and *in situ*. Some of these include the recovery of individuals with the gametic number of chromosomes, immediate expression of recessive genetic events, feasibility of getting homozygous individuals through either spontaneous or induced chromosome doubling, large numbers of microgametophytes available, high degree of developmental synchrony in certain species, and opportunity to treat several developmental stages.

In practice, however, the technology has not advanced sufficiently, even with model species as tobacco, to be used routinely. The benefits to be derived justify a research effort to develop one or more pollen embryogenetic assays for genetic toxicology. It would be especially beneficial to develop an assay in which the microgametophytes could be subjected to mutagens late in their developmental cycle, and the induced mutation could be assayed at the pollen grain level either prior to or at anthesis. Such an assay would provide a one-to-one relationship between the cells treated and those assayed. This would be a significant accomplishment in terms of a plant genetic assay. It would also permit the assaying of genetic events without numerous intervening developmental and metabolic processes that can either amplify or mask treatment effects. A judicious selection of plant species and stage of development might permit the assay to distinguish between chromosomal aberrations and point mutations.

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